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Expression of a fusion protein containing calf prochymosin B(1-161) and human proinsulin.
Tang J, Xue Y, Fan X, Fu Y.

2) Biochem Soc Trans 1991 Aug;19(3):248S
Secretion of a chymosin-insulin fusion in Trichoderma reesei.
Lawler SE, Pitts JE, Mantafounis D, Gill R, Uusitalo J, Penttila M.

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Chinese Journal of Biotechnology

Volume 9, Number 2

Expression of a Fusion Protein Containing Calf Prochymosin B(1-161) and Human Proinsulin

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The plasmid pJG202 containing Tac promoter, calf prochymosin B(1-161) gene and human proinsulin gene was constructed and transformed into *E. coli* JM105. The expression of the fusion protein consisting of 249 amino acids was controlled by IPTG and temperature, and the expressed protein was estimated to be 20-35% of the total cellular proteins by scanning of the SDS-PAGE gel stained with Coomassie brilliant blue R250. After CNBr cleavage, sulfitolysis, partial separation of the S-sulfonated human proinsulin and recombination of the disulfide bonds, human proinsulin with native properties, as evidenced by amino acid composition analysis, receptor binding and radioimmunoassays, could be obtained.

KEY WORDS: Human proinsulin; gene expression

Seventy years have passed since the first treatment of diabetes with insulin. In the major part of this period the only available way for obtaining insulin in from animal pancreas by purification. As the chemical structure of animal insulin is different from that of human insulin in some amino acid residues, the human body will generate some immunoresponses after long period of injection with animal insulin. It was impossible to obtain a large amount of human insulin until the introduction of recombinant DNA technique in the late 1970s. Two acceptable methods have been employed for the production of human insulin from microorganisms [1-3]. In the early 1980s this kind of work was also initiated in several laboratories in our country. However, due to problems in the systems, no satisfactory progress has been achieved. Obtention of even a small amount of purified human

proinsulin in the laboratory is still difficult [4,5]. Zhang Yuying et al. [6] reported the high level expression of calf prochymosin B gene in *E. coli*, under the control of Tac promoter. In this paper fusion of human proinsulin gene with part of the calf prochymosin B gene is described. After expression and processing of the fusion protein, the purified recombinant human proinsulin with native biological activity was obtained.

MATERIALS AND METHODS

Cell culture and plasmid

E. coli strain JM105 was cultured in LB medium. Plasmid pTaAC' was a gift from Dr. Yang Kaiyu of the Institute of Microbiology, Academia Sinica. It was constructed from pTaAC after removal of the 3'-terminal part of calf prochymosin B gene by EcoRI cleavage and recirculation with T4 DNA ligase, and contained Tac promoter and the 5'-terminal part of calf prochymosin B gene coding for the N-terminal peptide of 161 amino acids [6]. Plasmid pBCA was a gift from Dr. Shen Tongjian of the Institute of Biophysics, Academia Sinica. It contains a synthetic gene for human proinsulin prepared in Wu Ray's laboratory [7]. pUC19 was purchased from Sino-American Biotechnology Company.

Enzymes and reagents.

Restriction enzymes, T4 DNA ligase, and IPTG were purchased from Sino-American Biotechnology Company. Cyanogen bromide was obtained from Sigma. Human proinsulin standard as a gift from Dr. Tsou Chenlu of the Institute of Biophysics, Academia Sinica, was the recombinant product of E. Lilly Co. Human placenta membrane receptor was prepared as described elsewhere [8]. The kit for radioimmunoassay of insulin was from Beijing Xiyuan Biotechnology Center.

Construction of the expression vector

This was done according to Sambrook et al. [9].

SDS-Polyacrylamide gel electrophoresis

This was carried out as described [9] with 10% gel. After staining with Coomassie brilliant blue R250, the gel was analyzed by densitometric scanning.

Cyanogen bromide cleavage, sulfitolysis reverse phase HPLC and amino acid composition analysis

These were carried out as described previously [10,11]. Sephadex G50 column separation (fine, 2.5 × 100 cm) was carried out by eluting with 50 mM NH_4HCO_3 buffer. After reduction of the crude S-sulfonated human proinsulin in the denaturant, dialysis to remove the denaturant and excess reductant, the crude recombinant products were then obtained by oxidation according to the method described by Steiner and Clark [12].

Receptor binding and radioimmunoassays

This was carried out as described in our previous paper [11] for receptor binding assay. Radioimmunoassay was done mainly according to the instruction of the kit with some modification.

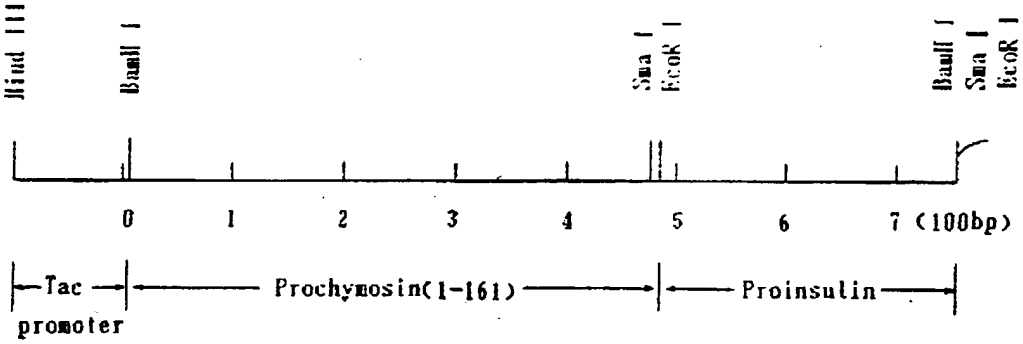

Fig. 1
Physical map of the fusion gene including Tac promoter, fusion protein gene and part of the pUC19 polylinker of pJG202.

RESULTS AND DISCUSSION

Construction and identification of the expression vector pJG202

Fig. 1 shows the construction of the expression vector pJG202. The Tac promoter and part of calf prochymosin B gene (1-161) was obtained from pTaAc' after digestion with HindIII and EcoRI. The human proinsulin gene was cut off from pBCA with EcoRI and BamHI. The plasmid pUC19 was cleaved with HindIII and BamHI. The expression vector pJG202 was then constructed by cloning of the above two small fragments into pUC19 in proper orientation with Tac promoter, part of the calf prochymosin B gene, and followed by human proinsulin gene.

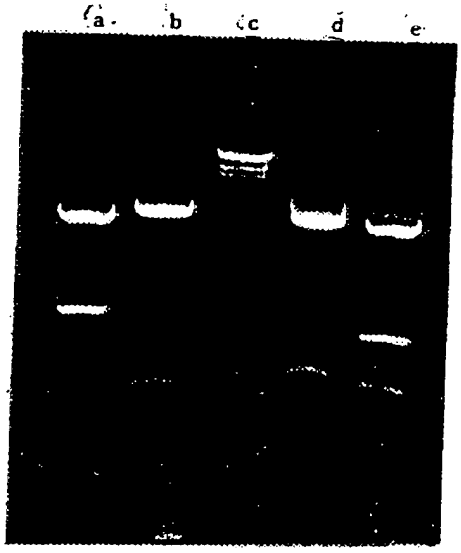

Fig. 2
Restriction map of pJG202.
a. BamHI; b. EcoRI; c. DNA digested by HindIII; d. SmaI; e. Hind III/EcoRI.

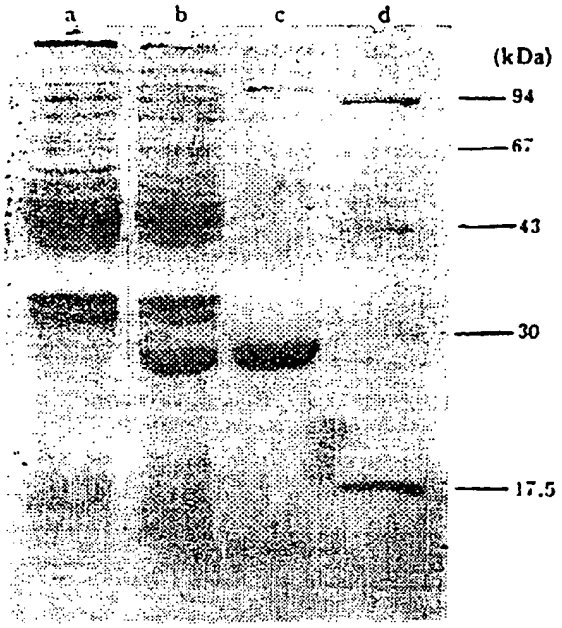

Fig. 3

SDS-PAGE analysis of the expression product of pJG202.

(a) Total protein of *E. coli* JM105 transformed with pUC19, (b) As (a) Transformed with pJG202, (c) Purified protein by sonication and centrifugation from (b), (d) Molecular weight marker (from large to small): Phosphorylase b, albumin, actin, carbonic anhydrase, TMV coat protein.

Fig. 2 demonstrates the restriction enzyme analysis of the constructed pJG202. The result is in agreement with that of the expected. There are two SD sequences at 3 and 10 base pairs respectively in front of the initiator coden of the fusion gene in pJG202. This factor gives positive effect on the high level expression of the gene. The reading frame at the junction between the calf prochymosin part and human proinsulin is correct with two additional amino acid residues, Arg(162) and Met(163). After cyanogen bromide cleavage at Met(163), human proinsulin with correct N-terminus could be generated. In the fusion protein with 249 amino acid residues, proinsulin constitutes almost one-third of the molecule, which is favorable for the cyanogen bromide cleavage and subsequent processing.

Expression of pJG202

The conditions for expression were almost the same as described elsewhere [6]. The wet cells were suspended in STET buffer in a ratio of 1/10(w/v) and sonicated. The lysed mixture was centrifuged to precipitate the inclusion bodies. Fig. 3 shows the SDS-PAGE analysis of the expressed product of pJG202. The expressed fusion protein molecule shows a molecular weight slightly smaller than 30kDa, in agreement to the expected 28kDa. It is obvious that the expression level is quite high. After this single step purification for the inclusion bodies, the expressed product attained good purity. The expressed protein was estimated to be up to 35% of the total cellular proteins by thin layer densitometric scanning of the SDS-PAGE gel stained with Coomassie brilliant blue R250. The purity of the purified inclusion bodies was as high as 81% quite favorable for subsequent studies.

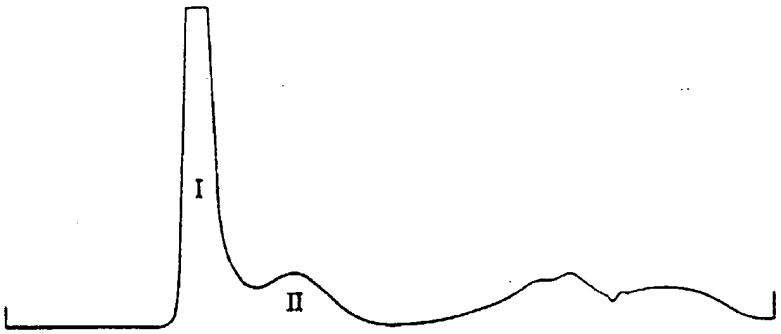

Fig. 4
Sephadex G50 separation of the purified inclusion bodies of pJG202 after CNBr cleavage and sulfitolysis. Fraction II contains the crude S-sulfonated human proinsulin.

Processing of fusion protein to human proinsulin

After cyanogen bromide cleavage, sulfitolysis, and separation on Sephadex G50 column, the crude S-sulfonated proinsulin could be obtained as shown in Fig. 4. The second peak in Fig. 4 contains the crude product while the first peak shows the existence of some large molecules owing to the incomplete cleavage with cyanogen bromide. The recombinant product with high biological activity could be obtained by the direct reduction and reoxidation of the crude S-sulfonated proinsulin [12].

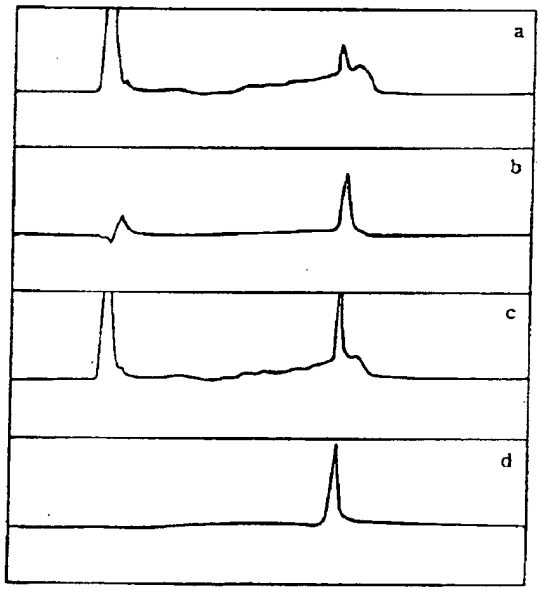

Fig. 5
HPLC analysis of the oxidation products of the partially purified S-sulfonated recombinant human proinsulin.
(a) Oxidation products alone, (b) Recombinant human proinsulin standard, (c) As (a) with the addition of (b), (d) Purified recombinant human proinsulin.

Table 1
Amino acid composition analysis of the recombinant human proinsulin.

	Expected	Found
Asp	4	5.5
Thr	3	3.3
Ser	5	4.3
Glu	15	14.7
Pro	3	3.3
Gly	11	11.0
Ala	4	5.1
Val	6	5.5
Ile	2	2.3
Leu	12	11.0
Tyr	4	3.3
Phe	3	3.0
Lys	2	2.0
His	2	1.9
Arg	4	4.3

Fig. 5 shows the reverse phase HPLC analysis of the recombinant product. A major peak could be seen at the same location by comparing with the recombinant human proinsulin standard. The corresponding peak was purified and a single peak in reverse phase HPLC was obtained as shown in Fig. 5d. The electrophoretic property of the present recombinant product was also the same as that of the standard (data not shown). The first relative big peaks in Fig. 5a and c are the result of salt components and some of the proteins in the void fractions. Table 1 shows the amino acid composition of the recombinant human proinsulin. The found value is quite in agreement with that of the expected.

Determination of the biological activity of the recombinant human proinsulin

The quantity of the product was measured by ultraviolet absorbance determination. The biological activities of the recombinant human proinsulin were then determined from different directions for their structures by receptor binding and radio immuno assays using Lilly's product as the standard. Figures 6 and 7 show the results of receptor binding assay and radioimmunoassay of the present recombinant product, respectively, indicating the existence of equal biological activity as compared with the standard human proinsulin.

Apart from the selection of the effective expression vector in the procedures for the recombinant human proinsulin, the other important factor is the design of the appropriate size of the fusion protein. The latter factor has some advantages not only for high level expression but also for purification after cyanogen bromide cleavage. If the number of methionine residues in the protein molecule is "n", the number of possible fragments of the incomplete cyanogen bromide cleavage is "m", then:

$$m = (n + 1)(n + 2)/2$$

If the number of possible fragments containing the needed peptide in the C-terminus, or complete cleavage fragments, is "p", then:

$$p = n + 1$$

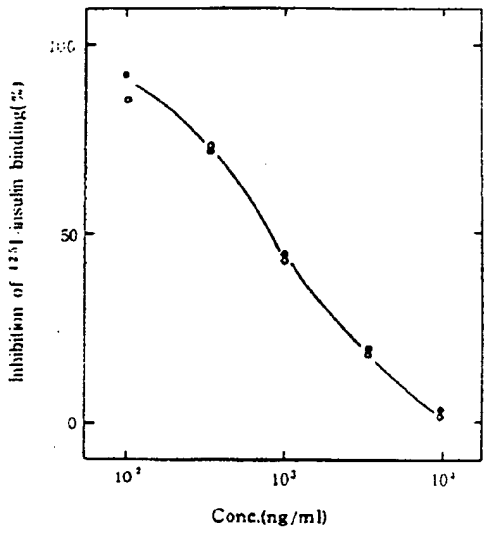

Fig. 6
Receptor binding assay of recombinant human proinsulin.
○ human proinsulin standard, ● purified product.

In the present expressed fusion protein with 249 amino acids, there are two methionine residues at positions 124 and 163 from the N-terminus. The number of possible fragments from incomplete cleavage is 6. The number of possible fragments containing proinsulin is 3. If completely cleaved, the number of fragments is also 3, thus making it easier for purification. The average amounts of methionine in ordinary protein is quite low. For example, take 10 protein molecules (calf prochymosin, bovine ribonuclease, human proinsulin, chicken lysozyme, bovine lactoglobulin, ferredoxin, TMV coat protein, horse cytochrome C, human carbonic anhydrase, and silk fibroin) there are 18 methionine residues within the total 1565 amino acids of the 10 protein molecules, corresponding to one

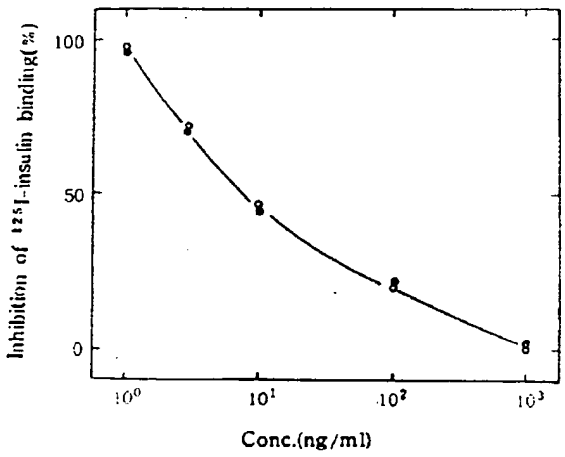

Fig. 7
Radioimmunoassay of recombinant human proinsulin.
○ human proinsulin standard, ● purified product.

methionine in about 80 amino acid residues. On the basis of the mentioned analysis above, for a fusion protein with 240 amino acids, the number of methionine residues is possible 3, the number of possible cleaved fragments is 10. If a larger protein size is chosen (e.g., 640 amino acids), the possible cleaved fragments would increase greatly (45 fragments), making it more difficult for the isolation and purification. As to the reported expression vectors, pWR450 and pWR590, the methionine residues in the fusion part of β-galactosidase together with one methionine residue coded by the initiator codon in front of the proinsulin gene, make the total numbers of methionine 14 and 16, respectively [13]. The possible numbers of cleaved fragments would be 120 and 153 would be respectively and possible numbers of proinsulin containing fragments 15 and 17, respectively. Even if cleaved completely, the fragment numbers are still 15 and 17, respectively, making it unfavorable for the purification of proinsulin from the complicated mixture [4]. It can be conceived that deletion by mutation of the methionine residue at position 124 in the fusion protein will make it easier for the following processing of the expressed product.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Gu Xiaocheng, Dr. Chen Zhangliang, Dr. Pan Naisui for their helpful support; Mr. Yuan Hongsheng for amino acid composition analysis; Dr. Hu Meihao for her valuable suggestions, also Dr. Yang Kaiyu, Dr. Shen Tongjian and Dr. Tsou Chenlu for their interest and encouragement.

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Secretion of a chymosin-insulin fusion in *Trichoderma reesei*

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The heterologous expression of polypeptide hormones in bacteria often results in the production of insoluble inclusion bodies and incorrectly formed disulphide bridges [1-3]. Chymosin is an aspartic proteinase which cleaves K-casein and initiating milk clotting [M_r 35,600; 323 amino acids, [4-7]. One of the natural variants of the enzyme chymosin A has been expressed in *Trichoderma reesei* utilising the cellobiohydrolase I promoter/operator [8]. Under inducing conditions chymosin A is made at levels of 20-40 mgs/l. We report here the construction of a fusion of a synthetic proinsulin gene with chymosin which is secreted under the control of the cbhI signal polypeptide of *Trichoderma reesei*.

The synthetic proinsulin gene was isolated as an *Eco* RI-*Bam*HI fragment, cloned into the vector pAMH104-E delta*Bam* HI containing the *cbh* I terminator and checked by agarose gel electrophoresis. This was confirmed by DNA sequencing using the M13 dideoxy chain termination method of Sanger [9]. After purification the *cbh* I terminator-insulin fusion was digested with *Eco* RI and dephosphorylated. The large fragment of the original chymosin expression vector was recombined with this material to produce the final construct pAMH104\PI (Figure 1.).

The expression plasmid pAMH104\PI was transformed into the *Trichoderma reesei* strain RUTC [10].

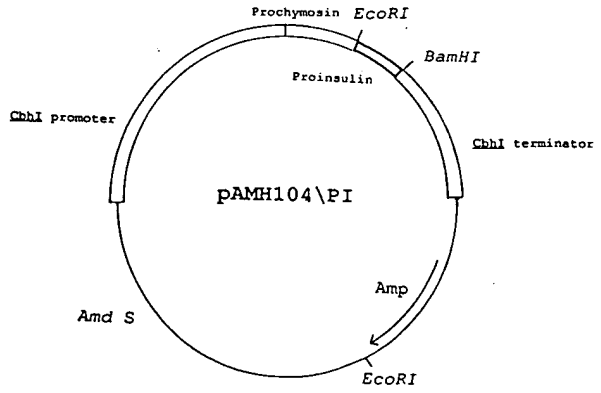

Fig. 1. The insulin-chymosin fusion vector containing the amdS gene which confers the ability to metabolise acetamide. The chymosin-insulin fusion is controlled by the cbh I promoter which is induced when *Trichoderma reesei*

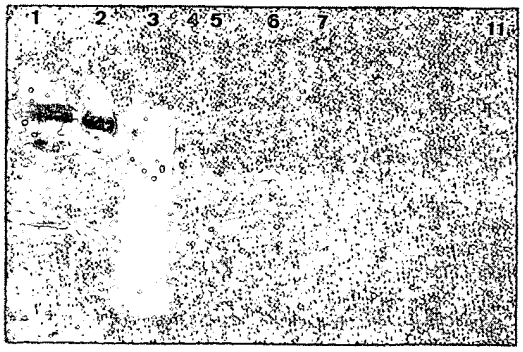

Fig. 2. Western blot analysis of *Trichoderma reesei* transformed with the chymosin-insulin fusion plasmid pAMH104/PI using a rabbit anti-chymosin primary antibody. Lane 1,õ 20ng of prochymosin; lane 2, 5ng of prochymosin; lane 3, supernatant from pAMH104 expressing chymosin A, lanes 4 to 11, supernatants transformed with pAMH104/PI. Lane 6 indicates a high level of expression

The colonies obtained after eight days were regrown twice on selective acetamide media and once on non-selective PD agar slants under day light illumination to induced spore formation. Spores were then regrown in liquid culture and assayed for expression by Western blot analysis (Figure 2.). The Western blot analysis indicate the formation of a fusion product which binds to both insulin and chymosin antibodies. Chemical cleavage studies are underway to produce material for biological analysis.

We thank Tom Blundell, Steve Wood, Helena Nevalainen, Sirkka Keränen and Matti Korhola for their help and encouragement. We thank EMBO for a Short Term Fellowship to JEP. We are also grateful for financial support from the AFRC, VTT, and a studentship to D.M. (SERC) and to S.E.L. (MRC).

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